Thermophilic *paenibacillus* Nitrogen Fixation bacteria increases cereal crops productivity

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Abstract: Biological nitrogen fixation (BNF) is a major contributor to the global nitrogen fixation and ranges between 50 and 60% of the total nitrogen fixed. In this work, 4 thermophilic bacterial strains that isolated from costal ridge of Mediterranean in Egypt were used. It was identified by 16 S rRNA as *Peanibacillus polymyxa* (*P. polymyxa*). Glutamine synthetase (GS), the key enzyme in the pathway of ammonia incorporation into amino acids by bacteria, was detected in these bacterial strains at approximately 46 KDa and proved by western blot reaction. Then, three field experiments were applied in three successive seasons (summer, winter, summer of 2010/2011) on cereal crops maize and wheat to evaluate the effect of our bacterial strains on crop productivity. The results showed that, plants inoculated with bacterial strains plus 75% (90 Kg) of the recommended dose of mineral fertilizers (MF) led to increasing the yield of maize crop by 7% and wheat crop by 13.7% more than plants that only treated with 100% (full dose) MF. Moreover, increasing the protein content in grains as a result of raising the rate of nitrogen inside it and this was obviously in the total protein profiling of seeds which new protein bands appeared in the region between 28 & 17 KDa in maize and at 36 KDa in wheat. In conclusion: The application of NF bacterial inoculums plus 75% MF are saving 25% (30 Kg/ fed) of MF and increasing the yield of crops, in addition, increasing the protein content in the grain.
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1.Introduction

Crop production systems in relation to N - supply is a basic plant biochemistry and physiology. Gene expression leads to protein synthesis and the formation of metabolic systems (David, 2002). The interactions between carbon dioxide (CO_2) and nitrate (NO_3^-) assimilation and their dynamics are of key importance for crop production. In particular, an adequate supply of NO_3^-, its assimilation to amino acids and their availability for protein synthesis, are essential for metabolism (David, 2002). Bacteria are the most dominant group of microbial diversity. *P. polymyxa* (formerly *Bacillus polymyxa*), a non pathogenic and endospore-forming *Bacillus*, is one of the most industrially significant facultative anaerobic bacterium. It occurs naturally in soil, rhizosphere and roots of crop plants and in marine sediments. It possess ecological and biotechnological importance, *P. polymyxa* has a wide range of properties, including nitrogen fixation, plant growth promotion, soil phosphorus solubilisation and production of exopolysaccharides, hydrolytic enzymes, antibiotics, cytokinin. It also helps in the enhancement of soil porosity (Sadhana, 2009)

Wheat *(Triticumaestivum)* is the most important cereal crop cultivated in Egypt. The local production of wheat is not sufficient for supplying the annual demands. Thus, to increase wheat production, more efforts should be directed to improve several agronomic practices, one example is the biofertilization. In addition, maize is the basis of human nutrition in some area in the world and is considered one of the most important cereal crops in Egypt where it is used mainly in animal and poultry feeding, (up to 75%) and in human nutrition for making bread. It is a major component in several important industries (corn oil, starch and fructose sugar). Corn is highly dependent on fertilization and is known to aggravate soil degradation more than some other crops. Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers as much as possible (Dilfuza, 2007). Bacterial inoculants are able to increase plant growth, speed up seed germination, improve seedling emergence, responses to external stress factors, protect plants against diseases (Lugtenberg *et al*., 2002). Biological nitrogenfixation is a major contributor to the global nitrogen fixation and ranges between 50 and 60% of the total nitrogen fixed (Cast, 1976). An understanding of ecological conditions affecting bacterial inoculants is important when introducing microbes for increasing plant growth and productivity. The bacterial inoculation has a much better

stimulatory effect on plant growth in nutrient deficient soil than in nutrient rich soil. *P. polymyxa* has promotion effect on plant growth and NPK uptake in nutrients deficient loamy sand soil (Dilfuza, 2007). In addition, Canway *et al*. (1988) reported that *P. polymyxa* plays an important role in rhizosphere of several crops. Plant growth promoting bacteria (PGPB) may be important for plant nutrition by increasing N and P uptake by the plants and playing a significant role as PGPR in the biofertilization of crops (Cakmakci *et al*., 2005). Vessey, (2003) reported that plant growth promoting bacteria have potentiality to convert nutritional elements from unavailable to available form through biological processes. The aim of the present work is, identifying four bacterial strains on molecular level (16S rRNA). Determination the suitable media to give the highest GS activity. Then, using that bacterial strains as inoculums in the field to test its effect on cereal crop's productivity.

2. Material and Methods

Bacterial strains:

Four: bacterial strains, SH7, SH8, SH9 and SH10 were isolated from Costal ridge of Mediterranean, identified as *P. polymyxa* by Bergey's manual and categories as thermophilic bacteria because it could grow in high temperature up to 70°C (Ibrahim *et al*., 2007). It were obtained from microbial molecular biology lab, Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt and used in this work. Two reference strains G & M (Gebreel *et al*., 2012) and one more *P. polymyxa* reference strain was obtained from Dr. Nabil Omar, SWERI, ARC, Giza, Egypt were also used in this work.

Bacterial identification:

Set of synthetic oligonucleotides homologous to broadly conserved sequences from 16S rRNA gene was used in 16S rDNA identification. The full-length 16S rDNA (1500 bp) were amplified from the strains by PCR using the universal primer pairs, forward P1 & reverse P6. P1 (5′- A GAG TTT GAT CCT GGT CAG AAC GCT-3′) (Edwards *et al*., 1989) corresponds to positions 8–37 and primer P6 (5′- T ACG GCT ACC TTG TTA CGA CTT CAC CCC-3′) corresponds to positions 1479–1506 in the *Escherichia coli* 16S rDNA (Yanagi and Yamasato 1993). The PCR product of the amplified 16S rDNA was purified from 1.2% agarose gel using the gel extraction kit (clean up 50 QUAquick spin columns kit, QIAGEN). Purified DNA (1.5 kb PCR product) were cloned into pGEM-T Easy cloning vector (Promega), and then, the constructed plasmids were transformed into *E. coli* JM109. The transformants were selected using the ampciline/blue/white colonies screening procedure. Plasmids containing 16S rDNA were isolated and purified (using plasmid extraction kit from Fermentas). The Purified plasmids were sent for sequencing (by Lab Technology Com.).

Testing for glutamine synthetase (GS) enzyme activity:

According to (Dean *et al*., 1977; Elliot, 1995; Gebreel *et al*., 2012) GS was assayed in crude extracts of bacterial isolates.

Immunoblot analysis:

First, total cellular proteins (crude extract) were prepared according to (Laemmli, 1970). **Then, the total protein of the bacterial cells were used** with a polyclonal antibody (raised against GS from *E. coli*., obtained from prof. Mike. Merrick, John Innes Centre, Norwich research park, Norwich, UK) to test for the presence of the enzyme (GS). The presence of the protein that represents the GS enzyme was detected in the crude extracts of bacterial cells by a western blot (immune-blotting) analysis (Lampel *et al*., 1994) (The kit used was: BS 393-RB, 5R, WESTERN Blot kit, BioBasic INC).

Field experiments on cereal crops to test the effect of bacterial inoculation on crop's productivity and to select the best bacterial strain from the four used strains. The strains SH7, SH8, SH9, SH10 were used in inoculation of field experiments and the strains M & G and *P. polymyxa* were used as a reference strains. The maize (variety 327 triple hybrid, obtained from Field Crop Research Inst., ARC) and wheat (variety sakha 93) experiments were conducted in three successive seasons; summer, winter and summer 2010 / 2011 at El-Nubariya site (Agricultural Research Station). To evaluate the role of bacterial strains in the crop productivity taking in consideration the growth of bacterial strains on the modified LB media according to (Gebreel *et al*., 2011).

Mineral Fertilizers (MF):

Ammonium nitrate and ammonium sulfate were used in maize and wheat experiments, respectively. 100% (120 kilogram) is the recommended dose. 75% is equal 90 Kg. All the bacterial strains that used as inoculums were combined with 75% MF. The bacterial cultures were carried on carrier (25% peat & 75% vermiculite)

Biofertilizers: the bacterial inoculants were applied by using seed coating techniques (nearly ~ 10^6 cells/one seed). In addition, two equal doses of liquid inoculants were added during the first and second irrigation period.

N P K uptake, soil biological activity as; nitrogenase, dehydrogenase and catalase and the microbial total count in the rhizosphere area were studied through these experiments:

Dehydrogenase activity (DHA) of root maize rhizosphere was estimated according (Casida *et al*.,

1964), **nitrogenase activity (N_2-ase)** was determined using GLC model HP6890 (Leth bridge *et al*., 1982 and Kitoh *et al*., 1993). The total bacterial counts were also estimated (data not showed). **Determination of catalase activity**: Catalase activity was determined by the method of (Cohen *et al*., 1970). The decomposition of hydrogen peroxide was measured by reacting with excess of potassium Tetraoxomanganate, KMnO4, and measured spectrophotometrically at 480 nm.

Random samples of grains and straw representing each replicate of all treatments were collected, oven dried, digested and assigned for analyzing N, P and K. **Total nitrogen** was determined using Kjeldahl method, according to (Chapman and Pratt, 1961). **Phosphorus** was determined colorimetrically according to (Jackson, 1976). **Potassium** was determined using flame spectrophotometer according to (Black, 1982).

3. Results and Discussion

Four bacterial strains SH7, SH8, SH9, and SH10 were identified as *p. polymyxa* by 16S rRNA. Gene cloning, sequencing and sequence blast (Zheng *et al*., 2000) revealed that all the four strains were *p. polymyxa*. Here are one example of our results, the tree in fig (1) shows the position of strain SH7 in the tree of *p. polymyxa* when the forward and reverse sequence were applied from the 1.5 kb 16S rDNA PCR product strain SH7 Forward seq. from 1.5 kb 16S rDNA product.

>EMBOSS_001
ATTGGGTACACCTTTAAGCTGGCTGGCTCCAA
AAAAGGTTACCCCACCGACTTCGGGTGT
TACAAACTCTCGTGGTGCTGACGGGCGGTGTG
TACAAGGCCCGGGAACGTATTCACCGCG
GCATGCTGATCCGCGATTACTAGCGATTCCAG
CTTCATGTAGGCGAGTTGCAGCCTACAA
TCCGAACTGAGAACGGTTTTATGAGATTAGCT
CCACCTCGCGGTCTTGCAGCTCTTTGTA
CCGTCCATTGTAGCACGTGTGTAGCCCAGGTC
ATAAGGGGCATGATGATTTGACGTCATC
CCCACCTTCCTCCGGTTTGTCACCGGCAGTCA
CCTTAGAGTGCCCAACTTAATGATGGCA
ACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTG
ACGACAACCATGCACCACCTGTCACTCTGCTC
CCGAAGGAGAAGCCCTATCTCTAGGGTT
TTCAGAGGATGTCAAGACCTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAAACCACATGCT
CCACCGCTTGTGCGGGCCCCCGTCAATTC
CTTTGAGTTTCAGCCTTGCGGCCGTACTCCC
CAGGCGGAGTGCTTAATGCGTTAACTTCAGCA
CTAAAGGGCGGAAACCCTCTAACACTTA
GCACTCATCGTTTACGGCGTGGACTACCAGGG
TATCTAATCCTGTTTGCTCCCCCACGCT
TTCGCGCCTCAGTGTCAGTTACAGACCAGAAA
AGTCGCCTTCGCCACTGGGTGTTCCTCC
ATAATCTCTACGCATTTCACCGCTACACATGG
AATTTCCACTTTTCCTCTTCTGCACTCA
AGTCCTCCCCAGGTTTCCCAATGAACCCTCCA
CGGTTTGAGCCGTGGGGCTTTTCCACCA
TCATAACTTAAGAAAACCCACCTTGCGCGCCG
CTTTACGGCCCAATTAAATTTCCGGGAA
TAACG

strain SH7 Reverse seq. from 1.5kb 16S rRNA product

>EMBOSS_001
CGATTGGGCCAAATCATGCAAGTCGAGCGAA
TGGATTAAGAGCTTGCTCTTATGAAGTTA
GCGGCGGACGGGTGAGTAACTCGTGGGTAAC
CTGCCCATAAGACTGGGATAACTCCGGGAAA
CCGGGGCTAATACCGGATAACATTTTGAACCG
CATGGTTCGAAATTGAAAGGCGGCTTCGGCTG
TCACTTATGGATGGACCCGCGTCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCAAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGCTTTCGGGTCGTAAAACTC
TGTTGTTAGGGAAGAACAAGTGCTAGTTGAAT
AAGCTGGCACCTTGACGGTACCTAACCAGAA
AGTCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAAT
TATTGGGCGTAAAGCGCGCGCAGGTGGTTTCT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCG
TGGAGGGTCATTGGAAACTGGGAGACTTGAG
TGCAGAAGAGGAAAGTGGAATTCCATGTGTA
GCGGTGAAAATGCGTAGAGATATG

Assay of glutamine synthetase (GS) enzyme activity

Assimilation of ammonium is a critical biochemical process for plant growth and development. GS functions as the major assimilatory enzyme for ammonia derived from nitrogen fixation and nitrate and ammonia nutrition. One specific important feature of GS is its high affinity for ammonia and, consequently its ability to incorporate ammonia efficiently into several organic configurations. It also assimilates ammonia released from photorespiration and break down of proteins (De Bashan, 2008). The data in **fig 2** showed higher activity of GS in the presence of the Mg^{++} divalent metal ion than with Mn^{++} ion in all the strains that grown in LB media or in LB with additives (NaCl 1.5 g/L, Glycerol 7ml % and Asparagine 0.4 % W/V). Fig 2 showed also, that the GS activity is higher in the strains that grown in LB + additives and varied among the bacterial strains. It was the highest in strain SH9 followed by SH10 grown in LB plus additives in presence of Mg^{++} ions. These data agreed with that

published by (Yuan *et al*., 2001). GS from *Synechococcus* RF-1, Mg^{++} was the most effective metal ion for its activity, when they used Mg^{++}> Co^{++}>Mn^{++}. On the other hand, GS(s) from *Prevotella ruminicola* 23 was observed only in the presence of Mn^{++} metal ion while not with Mg^{++}, Cu^{++}, Co^{++}, or Ca^{++} (Kim *et al*., 2012). **In fig 3**, the western blot analysis of GS enzyme, the GS of the 4 bacterial strains and the reference strain M showed sharp bands (fig 3: b) at approximately 46 KDa. Annick *et al*., (1992) published the immunoblot of GS enzyme from fungus *Laccarialaccata*, it was under the molecular mass 43 KDa, and this almost agree with the GS from the strains used in this work. While, GS from *Lacto bacillus* was ~ 56 KDa (Veera *et al*., 2012).

Field experiments:

Three successive field experiments were conducted at El-Nubaryia site. The soil used in planting the maize and wheat experiments (summer/winter/summer 2010/ 2011) were analysed before inoculation with the different bacterial strains for: pH (8.36), electric conductivity (1.40 ds/m), Organic matter (0.26%), Soluble cations mmol/L (Ca^{++}[4.90], Mg^{++}[6.60], Na^{+}[3.90], K^{+}[2.80]), Soluble anions mmol/L (CO_3^{--}[0.0], HCO_3^{-}[4.30], CL^{-} [8.20], SO_4^{--}[2.70]), available macro nutrients mg/kg (N [35.8], P [3.9], K [127.13]), and mechanical analysis (sand% [49.72], silt% [28.93], clay% [21.35] & texture [sand / clay / loam]). Audjima *et al*., (2014) did the same analysis when they were preparing the soil before planting.

Enzymatic activities:

There were three determinants of three important enzymatic activities in the rhizosphere soil of maize plant during the growth periods. These enzymes were: Nitrogenase, Dehydrogenase and Catalase. The data showed increase in these enzymes in the rhizosphere of inoculated plants with bacteria agreed with (García-Gil *et al.,*2000; Masciandaro *et al.,* 2000; Zhang *et al.,* 2009), who published that dehydrogenase and catalase activities in soil are mainly associated with soil microbes.

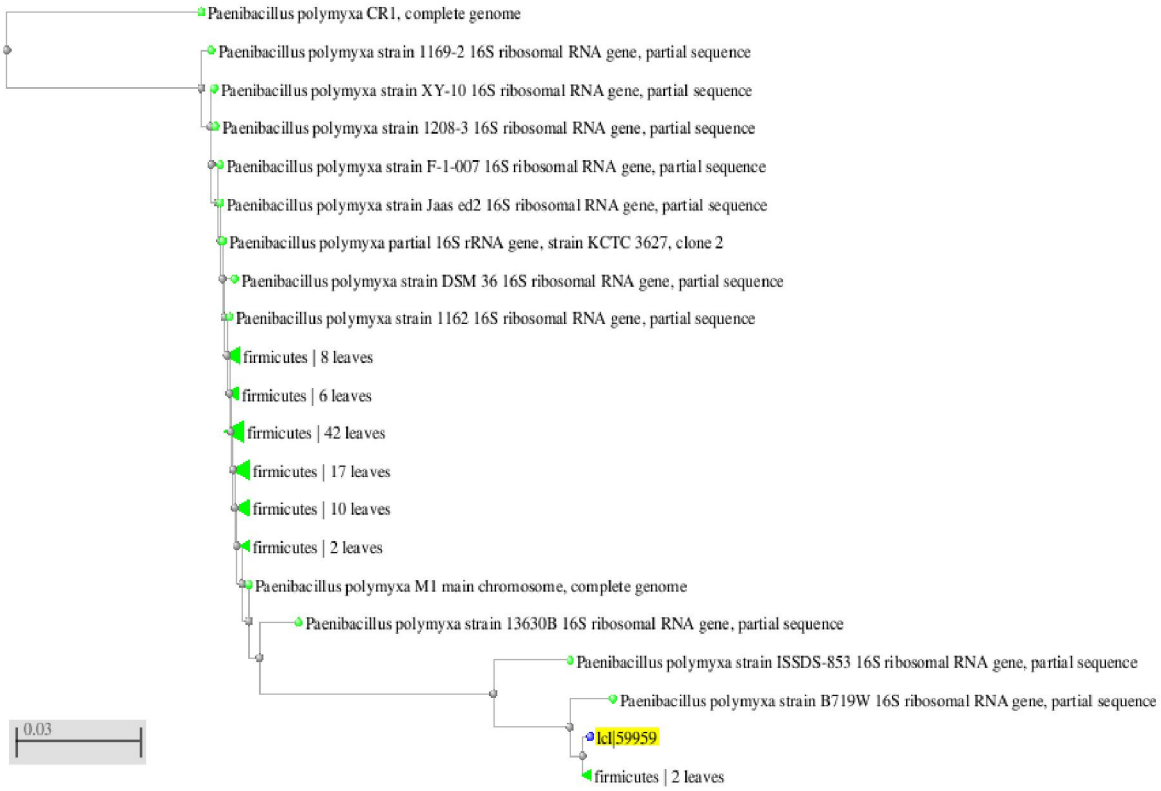

Fig 1: The taxonomic tree of strain SH7. 172 blast hits on the forward seq. from strain 7 and 170 blast hits on the reverse seq. The yellow 1c1l 59959 represents stain SH7

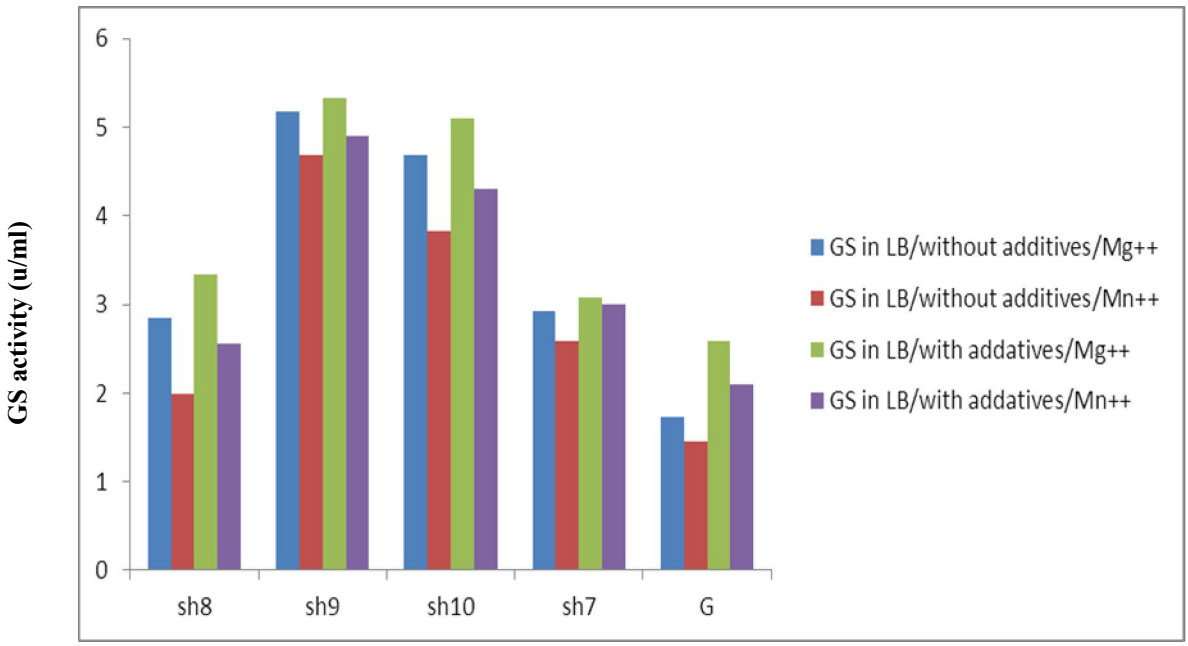

Fig 2: The chart shows the values of Glutamine synthetase (GS) activity. It shows the comparison between the use of the two ions Mg^{+2} & Mn^{+2} and between the LB free and that with additives

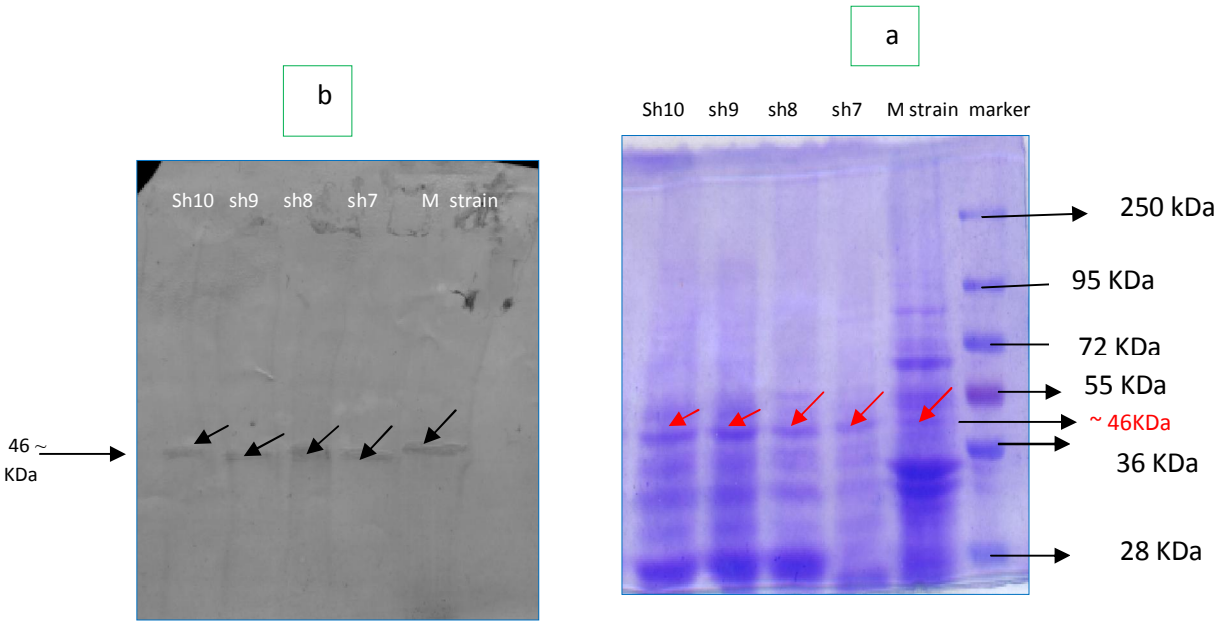

Fig 3: **(a)** showed SDS-PAGE for total cellular protein from the four strains SH7, 8, 9, and 10 and from the reference strain M. **(b):** the western blot of the same SDS-PAGE in (a), sharp bands ~ 46 KDa bound with the antibodies that raised in rabbit against GS enzyme.

Nitrogenase activity

The nitrogenase activity may be attributed to the effect of exudation of carbon compounds that have special importance to the growth of nitrogen fixing microorganisms which produce growth regulating compounds (indole acetic acid, gibberellins and cytokines-like substance) (Tien *et al*., 1979) and that explain the difference in nitrogenase activity between the inoculated plants with bacteria plus 75% MF and that treated only with MF (treatments 14 & 15 in table 1, column 1). The data showed the difference between the bacteria that grown on LB media and that grown on LB + additives. The data at the second month of planting, showed that plants inoculated with

strain 7 gave the highest nitrogenase activity (785 umol C2H4/hr/100 g soil) > strain 8 (780 umol C2H4/hr/100 g soil) > strain 10 (360 umol C2H4/hr/100 g soil) (table 1, column 1) compared with MF 75 % & 100 % (95 and 45 umol C2H4/hr/100 g soil respectively). Audjima *et al*., (2014), found that nitrogenase activity of the soil gave high nitrogenase activity at the 4 and 6 week when inoculated with *azotobacter* and *azospirillum* and planting maize.

Dehydrogenase enzyme

Releases hydrogen ions in the rhizosphere, resulting in formation of carbonic acid to decrease pH value. This process increases nutrient uptake and availability of the nutrient in rhizosphere, which, in turn, supported higher plant growth and yield (Omar and Ismail, 2002). The data revealed that some inoculated treatments with the different bacterial strains gave higher dehydrogenase activity than the not inoculated treatments specifically at the 2nd month of inoculation (table 1, column 2). There were also differences between the activities of the different bacterial strains.

Catalase enzyme activity

May be related to the metabolic activity of aerobic organisms and has been used as an indicator of soil fertility. Catalase (EC 1.11.6) is an iron prophyrin enzyme which catalyses very rapid decomposition of hydrogen peroxide to water and oxygen. The enzyme is widely present in nature, which accounts for its diverse activities in soil. Catalase activity alongside with the dehydrogenase activity is used to give information on the microbial activities in soil. The data of catalase activity presented in (table 1, column 3).

Table 1: Potential Nitrogenase activity as µmoles C_2H_4/ hour / 100g soil, Dehydrogenase activity as µg TPF / g soil and Catalase activity µmoles O_2 / g soil in the rhizosphere soil of maize plant at El-Nubaryia site 2010

No.		Treatments	Potential nitrogenase activity µmoles C_2H_4/hr/100 g soil	Dehydrogenase activity as ug *TPF / g soil	Catalase activity umoles O_2 / g soil
1	A	Strain 8	780	589	33
2	A	Strain 9	249	328	32
3	A	Strain 10	202	438	55
4	A	M	134	379	56
5	A	G	89	196	51
6	A	Strain7	785	245	46
7	B	Strain 8	117	181	51
8	B	Strain 9	126	254	29
9	B	Strain 10	360	221	29
10	B	M	194	247	30
11	B	G	100	148	28
12	B	Strain7	184	172	27
13		*P. polymyxa* reference str.	100	150	28
14		Inoculation with 75% MF	95	207	26
15		Inoculation with 100% MF	45	143	27

In table 1: **A** refers to treatments 1 - 6 which the bacterial strains were grown on LB. **B** refers to treatments from 7 - 12 which the bacterial strains were grown on LB + additives. * TPF: TTC (tetra zollium chloride / yellow color) reduced to TPF (Tetraformazan / red color) H is released by the activities of microorganisms. Table 1 (column 1) showed that all the inoculated treatments plus 75% MF gave higher nitrogenase activity than treatments treated with MF (treatments 14 & 15). Strain 7 gave 17.5 time higher than that treated with 100% MF, Column 2 in the same table, showed the dehydrogenase activity in the rhizosphere soil of maize. Strains 8, 10, M and 9 showed the highest dehydrogenase activity (4 times, 3 times, 2.65 times and 2.29 times more, respectively) compared with 100% MF. Column 3 showed the catalase activity. The bacterial inoculation plus 75% MF gave 2 times activity higher than fertilization with 100% MF (strains 10, 7, 8 and 9)

Determination of basic nutrients N P K in the rhizosphere soil and shoots of maize plants:

Measurement of biological nitrogen fixation activity can be used to characterize the changes in the soil properties and quality as a consequence of the farming system. BNF convoy to synthesis of substances that can be assimilated directly by plants. **Nitrogen (N)** are required for the formation of amino

acids and protein and it is limiting nutrient for plants. It is followed by **phosphorous (P)**, the second essential nutrient necessary for plants. P as chemical fertilizer become insoluble and unavailable to plant. then the use of P- solubilizing bacteria represents green substitute for chemical P (Paula *et al*., 2015). **Potassium (K)** is the third essential nutrient necessary for plant growth. In this work **NPK in (ppm)** were determined in soil of maize plants. the data showed (table 2) the inoculated treatments with bacterial strains plus 75 % MF gave higher N, P and K values (column 1, 2, 3) than that treated with only 75 % & 100 % MF. NPK % were also determined in shoots (table 2, column 4, 5, 6) of maize plants. The sampling in the second month of planting showed the highest values in both soil and shoots samples. In agreement with our results (Marihus *et al*., 2012) recorded a significant increase in concentration of N (4%), P (30%), K (17%) of maize plants as a result of combination of bacterial inoculation with NPK.

Dry weight of maize shoots:

plant development was enhanced by combination with plant growth promoting bacteria (PGPB) which resulted in significant increases in dry mass of shoots (7%) compared with non-inoculated plants, reported by (Marihus *et al*., 2012). The data in (table 3) revealed that in the second month of planting, all the bacterial strains plus 75% MF gave higher dry wt. than samples treated only with MF. Inoculation with strain 7 gave 19% increase in comparison to 75%MF (treatment no.14 in table3) and 41% increase in comparison to 100% MF (treatment no. 15 in table 3). Followed by strain 8 (13.9% & 37.5% increase over 75% & 100% MF respectively). *P. polymyxa* the reference strain gave 37.5% & 54.7% increase over the 75 & 100% MF respectively.

Crop's productivity:

Thus, bacterial biofertilizers can improve plant growth and crop's productivity through many mechanism; synthesis of plant nutrients, mobilization of soil compounds to be available for the plant, protection of plants and defense against plant pathogen (Paula *et al*., 2015). Inoculation of cereal seeds with NF bacteria decrease MF needs and improve crude protein (Reddy *et al*., 2003). Pahwa (1986), reported that, the use of *Azospirillum* inoculant reduced N needs 15 Kg N/ha^{-1}. Application of 80 Kg N plus seed inoculation with *azotobacter* gave a similar yield to that of an application of 120 Kg N/ha^{-1} alone (Agrawal *et al*., 1996). **In table 4**, **for plant heights/cm** (column 1), samples that inoculated with bacterial strains plus 75% MF showed: (strain 8 > strain 10 > strain7 & strain 9) increase in plant heights 10% over 100% MF and 12.2% over 75% MF, 8.6% & 10.6% over 100 & 75% MF and 6.9% & 8.8% over 100% & 75% MF respectively. **For wt. of 1000 maize seed/g** (column 3), samples inoculated with strains 8 & 10 gave increase in wt. of seeds 7% & 6.27% over 75 & 100% MF respectively. In agreement with our results, (Milosevic *et al*., 2012) recorded an increase 16% in 1000 - seed weight of wheat when inoculate the wheat plants with *Azotobacter chroococcum*. **For weight of yield**, all the samples inoculated with bacterial strains plus 75% MF gave increase in yield higher than that treated only with 75% MF, and the strains showed variations between each other. Strain *P. polymyxa* showed 7% increase & strains 8 and 10 showed 5% increase over the 100% MF. The chart in fig 4 shows the increase of the yield over the 100% MF (it represents column 4 from table 4, wt. of yield ton / hectar).

The data in table 2 showed the NPK in ppm in soil. Samples treated with strain 9 gave 34% **N** increase over that treated with 75% MF and 85% over that treated with 100% MF. Followed by strains 7 & 8 (gave 32% & 84.5% increase over 75 & 100% MF, respectively). Strains 10 & 9 gave 48.7% **P** increase than 75%MF and 43.5% P increase than 100% MF. Followed by strains 7 & 8 that gave increase in P (37.5% & 31.25% than 75 & 100% MF respectively). For **K**, strain 9 gave 8.1% increase than 75 & 100% MF, followed by strain7 that gave increase 6.2% than 75 & 100% MF. Nitrogen % in the plant shoots treatment of strains 9, 10 and strain 7 gave the highest value of nitrogen, and in phosphorus the bacterial strains 8, 9, 10, M and 7 showed high percentage of phosphorus. On the other hand, all the treatments that inoculated with bacteria gave the highest value of potassium in plant shoots.

Estimation of nitrogen (N)% in the grains after harvest of the maize plants, the data showed that the percentage of the nitrogen was higher in the treatments inoculated with strains, SH9, 10, G and strain *P. polymyxa*, (1.6 %, 1.6 %, 1.8 %, 1.5 % respectively) while that was treated with only 75 % and 100 % MF was (1.3 % and 1.4 % respectively). Thus, the total nitrogen increased by 18.75% to 27% in grains as a result of inoculation with bacterial strains using the equation (N % × 6.25 = protein content, the factor 6.25 is changing by the type of grain, according to the method, AOAC, 2005). **Total protein pattern of maize seeds (fig 5):** The protein patterns of the treatments showed a change in the protein bands in the region between 28 KDa and 17 KDa. One can observe the difference between the samples that inoculated with bacteria plus 75 % MF and that treated with only 75 % and 100 % MF (fig 5, lanes: 14, 15, 29, 30, 44, 45). This clarify the increase of protein in the grains.

Table 2: Determination of the available basic nutrients N P K (nitrogen - phosphorus - potassium) in ppm in the rhizosphere soil and NPK % in the shoots of maize plants at El - Nubaryia site 2010

No.		Treatments	NPK in ppm in the rhizosphere soil of maize plants			NPK% in the shoots of maize plants		
			N ppm/ soil	P ppm/ soil	K ppm/ soil	N %	P %	K %
			2nd period	2nd period	2nd period	2nd period	2nd period	2nd period
1	A	Strain 8	66	32	219	1.58	0.28	2.74
2		Strain 9	87	39	216	2.66	0.30	1.95
3		Strain 10	61	21	224	1.29	0.26	1.61
4		Strain M	62	23	237	1.32	0.37	1.47
5		Strain G	68	23	234	1.25	0.21	1.59
6		Strain 7	57	32	218	1.22	0.29	1.61
7	B	Strain 8	84	22	229	1.75	0.26	1.55
8		Strain 9	62	26	244	1.10	0.14	1.61
9		Strain 10	57	39	233	2.60	0.30	1.81
10		Strain M	38	22	222	1.54	0.26	1.89
11		Strain G	86	29	214	1.08	0.11	1.79
12		Strain 7	84	29	239	2.0	0.25	1.70
13		*P. polymyxa*	74	26	249	1.26	0.12	1.81
14		Treatment inoculated with only 75% MF	57	20	225	1.43	0.22	1.48
15		Treatment inoculated with only100% MF	13	22	224	1.04	0.11	1.70

In table 2: A refers to treatments 1- 6 strains were grown on LB. B refers to treatments from 7 - 12 where bacterial strains grown on LB + additives

Table 3: Effect of bacterial strains that were grown on different growth media on dry weight of maize shoots (g/dry wt.) during the different growth periods at El-Nubaryia site 2010

No.		Treatments	1st period 7/2010	2nd period 8/2010	3rd period 9/2010
1	A	Strain 8	39.96	322.9	203.1
2		Strain 9	38.83	288.1	273.1
3		Strain 10	90.0	246.7	325.6
4		M	42.2	220.4	222.3
5		G	39.4	278.9	217.1
6		Strain7	54.6	343.4	203.9
7	B	Strain 8	23.9	290.0	302.4
8		Strain 9	22.15	334.6	138.8
9		Strain 10	47.7	289.5	308.7
10		M	26.8	310.7	142.6
11		G	26.9	313.1	154.2
12		Strain7	22.3	316.3	369.1
13		*P. polymyxa* reference str.	30.1	445.6	362.7
14		Inoculation with 75% MF	29.2	278.1	268.0
15		Inoculation with 100% MF	30.2	201.7	276.4

Table 4: :Effect of bacterial inoculation and 75% mineral fertilization on the maize crop and yield components compared to full mineral fertilization without inoculation with bacteria at Nubaryia site 2010.

No.		Treatments	Plant height /cm	Wt. of Maize Seeds/g /ear	Wt. of 1000 Maize seed /g	Wt of yeild Ton/ hectar	Wt. of yeild Ardab/ feddan
1	A	Strain 8	262	206.7	342.8	10.97	21.94
2		Strain 9	260	243.7	368.4	11.235	22.47
3		Strain 10	247	199.5	326.2	10.892	21.78
4		M	247	221.8	355.5	10.771	21.54
5		G	252	228.4	373.9	11.421	22.84
6		Strain7	232	212.4	372.9	11.214	22.42
7	B	Strain 8	270	238.7	398.1	12.042	24.08
8		Strain 9	260	209.1	342.4	11.085	22.17
9		Strain 10	265	243.4	396.8	11.514	23.02
10		M	235	208.5	386.3	11.128	22.26
11		G	250	208.1	359.5	11.042	22.09
12		Strain7	260	228.6	358.7	11.421	22.84
13		*P.polymyxa* reference str.	217	219.0	394.1	12.285	24.57
14		Inoculation with 75% MF	237	217.6	369.9	10.800	21.60
15		Inoculation with 100% MF	242	213.3	373.1	11.471	22.94

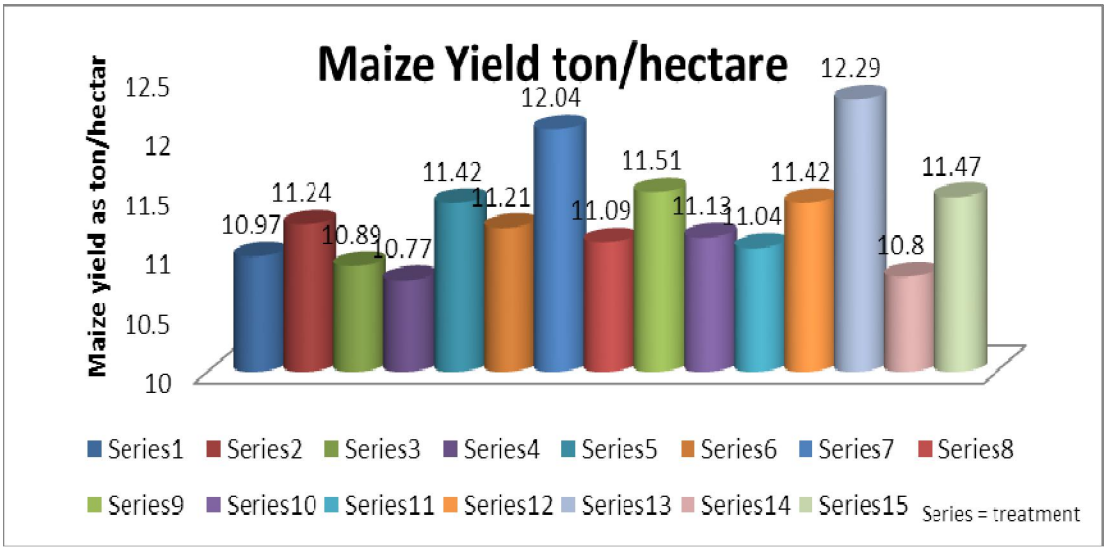

Fig4: Inoculation of different Bacterial strains for maize plants as compared with not inoculated treatments at El-Nubaryia site 2010. (The fig represents weight of yield ton/hectar from table 4).

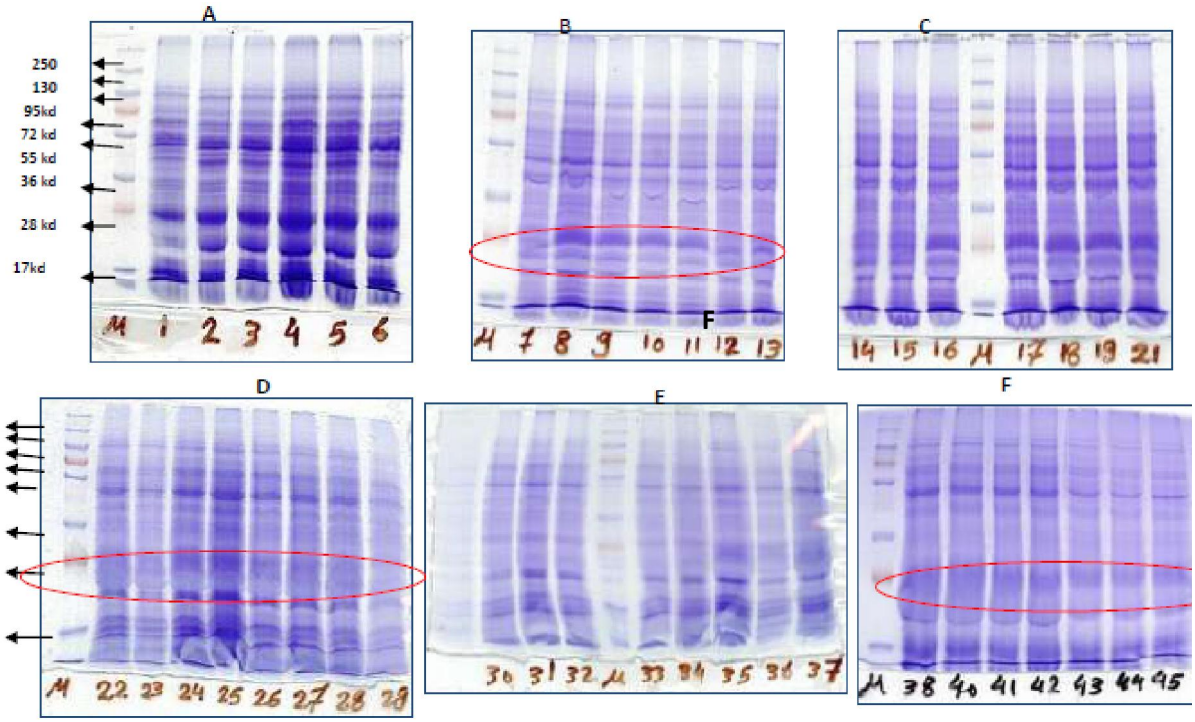

Fig 5: Total protein pattern of maize seeds

Treatments that inoculated with bacterial strains 8, 10 and B. polymyxa gave the highest yield as compared with treatment 14 (not inoculated with bacteria and used 75% mineral nitrogen fertilizer). They gave increase 11.6%, 6.7% and 13.7%.

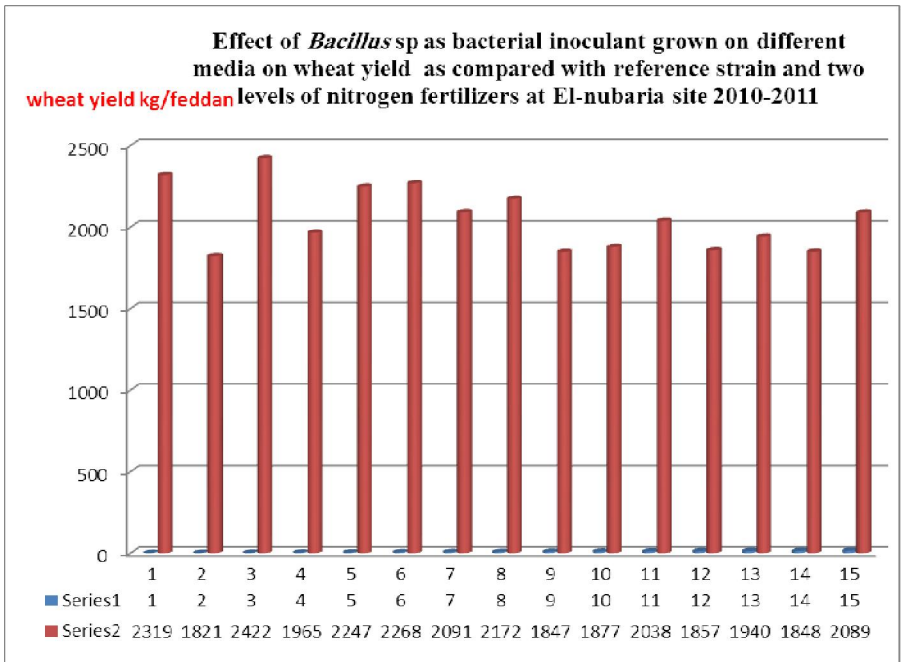

Fig 6: Effect of *P. polymyxa* as bacterial inoculants grown on different media on wheat yield as compared with reference strain and two levels of nitrogen fertilizers at El-Nubaryia site 2010-2011

In ig 6: from 1 to 6 are samples inoculated with bacterial strains plus 75% MF (*Bacillus* strains grown on LB media) -- from 7 to 12 are samples inoculated with bacterial strains plus 75% MF (the same strains were grown on LB media with additives). 13, is the reference strain *P. polymyxa*-14, sample treated with75% MF.15, sample treated with 100% MF.

1= SH8, 2= SH9, 3= SH10, 4=SH7, 5= G, 6= M (grown on LB media)

7= SH8, 8= SH9, 9= SH10, 10=SH7, 11= G, 12= M (grown on LB + additives)

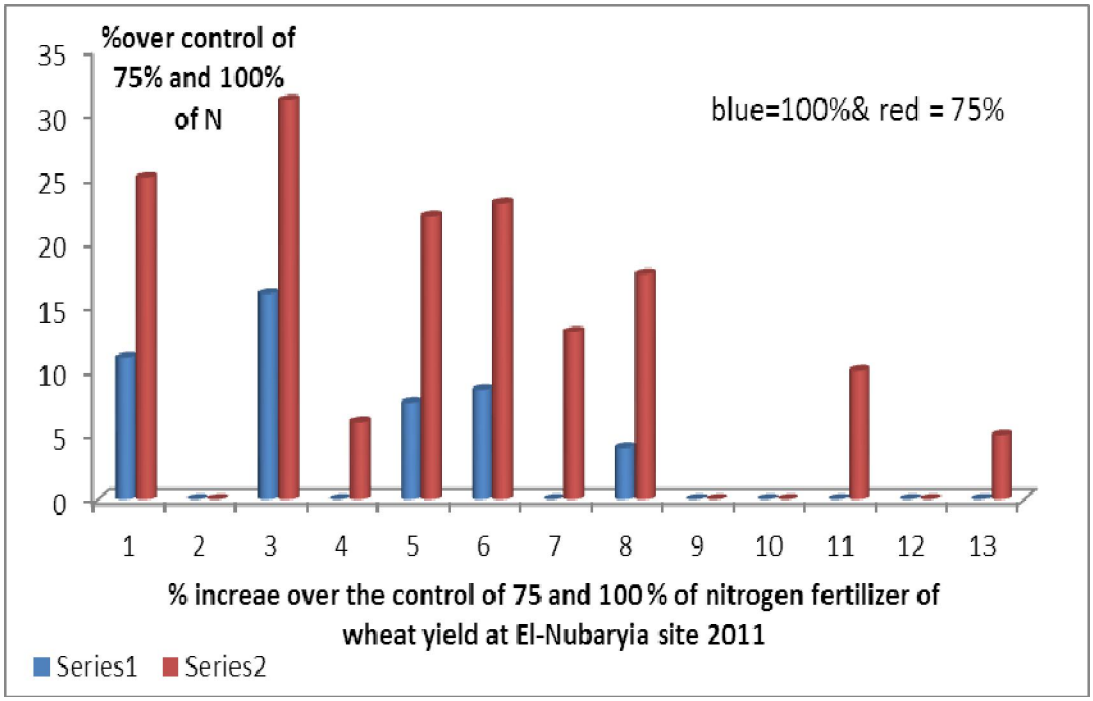

Fig 7: The productivity of the wheat yield as a result of bacterial inoculation + 75 % MF compared with fertilization with only 100% MF (treatment no. 15)

Fig (5) shows the total protein pattern from the seeds of the maize yield. The lanes 1 to 45 in A, B, C, D, E and F represent protein samples from 3 replicates of the treatments 1 to 15. Thus, 1 – 15 are the treatments used in the experiment. 16 -30 are the 2nd replicate of the same treatments. 31 – 45 are the 3rd replicate of the treatments. M: is protein marker, its bands from up to down are: 250 KDa, 130, 95, 72, 55, 36, 28, and 17 KDa.

The wheat experiment (winter 2010-2011 Nubaryia site, Wheat variety: Sakha 93)

The same experiments that have been done with maize were conducted on wheat and the effect of bacterial inoculation plus 75% MF on wheat was almost the same as on maize. So that, only the tables of the yield and protein profile are presented here.

As shown (in fig 6), all the samples inoculated with bacterial strains plus 75% MF gave increase in the yield over the samples that only treated with 75% MF. Samples inoculated with the bacterial strains SH10, SH8, G, and SH9 gave increase in the wheat yield over the sample treated with 100% MF by 13.7%, 10%, 7% and 4% respectively. Fig. 7 clarified the data in fig. 6 showing how are the wheat yield increased over the control (100 % MF). The treatments no. 1, 3, 5, 6, and 8 showed differences in the yield ranged between 4 to 13.7% increase over the control (100% MF). Thus, there was increase in the yield as a result of bacterial inoculation plus 75% MF specifically the treatment no. 3 that was inoculated with the bacterial strain **SH10**. These results revealed the possibility of saving 25% of MF (30 Kg / fed) and raising the yield to about 13.7%. These data assured that obtained with previous maize experiment. In a similar results (Milosevic *et al*., 2012) reported that, they obtained 74% increase in wheat yield when used 50 Kg ha^{-1} of urea and inoculation with *Azotobacter chroococcum*. This were varied from cultivar to other. Concurrence with (Minaxi *et al*., 2013), who published their attempt to explore the effect of mixed microbes to inoculate wheat to get the best yield. A significant increase in yield and nutrient uptake of wheat plants were noticed and wheat yield recorded increase by 92.8%. **From** all the results of the two experiments of maize and wheat, strain SH 7 and strain SH10 showed good results. Thus, SH7 and SH10 were chosen to complete our work on them, and the third maize experiment was conducted using only SH7 & SH10 with the reference strain *p. polymyxa*.

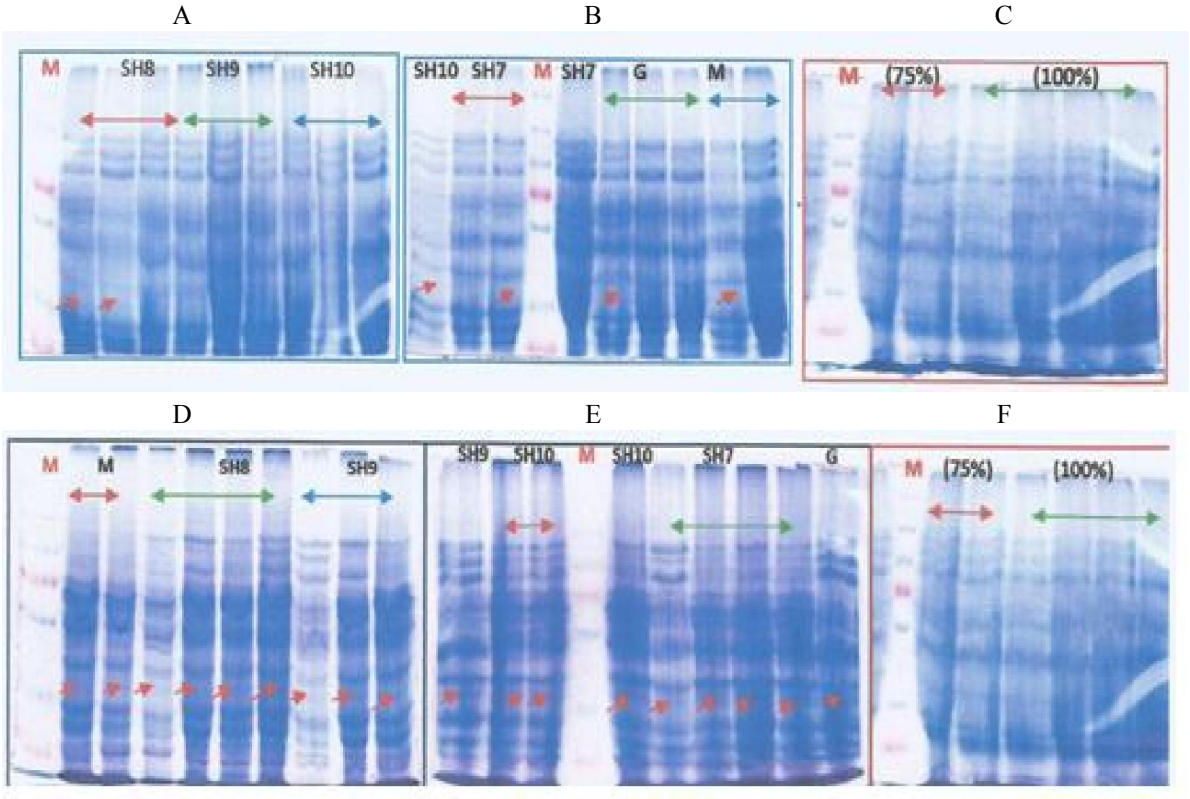

Fig 8: Total cellular protein from seeds of wheat yield

Gel A: M is prestained protein marker ladder (Fermentas), its bands from up to down are; 250, 130, 95, 72 (red), 55, 36, 28 (red), 17 and 11 kDa. 3 replicate from SH8, 3 replicate from SH9, and 3 replicate from SH10. **Gel B:** SH10, 2 replicate from SH7, **M**: marker**,** SH7, 3 replicate from sample G, 2 replicate from sample M. **Gel C: M**: marker, 2 replicate from samples treated with 75% mineral fertilization, and 4 replicate from samples treated with 100% mineral fertilization. All samples in both gels **(A & B)**; the bacterial strains were grown on LB media. when compared with gel **(C)** that contains samples treated with 75& 100 % MF (no bacterial inoculation). there are protein bands appeared in the region referred with red arrows (slightly above 36 kDa), while are not found in the samples in gel C. **Gel D: M:** prestained protein marker, followed by 2 replicate from samples inoculated with strain M, 4 replicate from samples inoculated with strain SH8, 3 replicate from samples inoculated with strain SH9. **Gel E:** SH9, 2 replicate from SH10, **M:** marker, SH10, 4 replicate from SH7, sample G. **Gel F:** the same **gel C**, we put it here for comparison. Both gels **D & E**; the bacterial strains were grown on LB media + additives (NaCl 1.5 g/L, glycerol 7ml % and asparagin 0.4 % w/v). When compared with gel **(F)** that contains sample treated with 75 & 100% MF (no bacterial inoculation), there are protein bands appeared in the region referred with red arrows (slightly above 36 kDa), while are not found in **gel F**. The appeared protein bands are sharper and heavier than that appeared in the gels **A & B.**

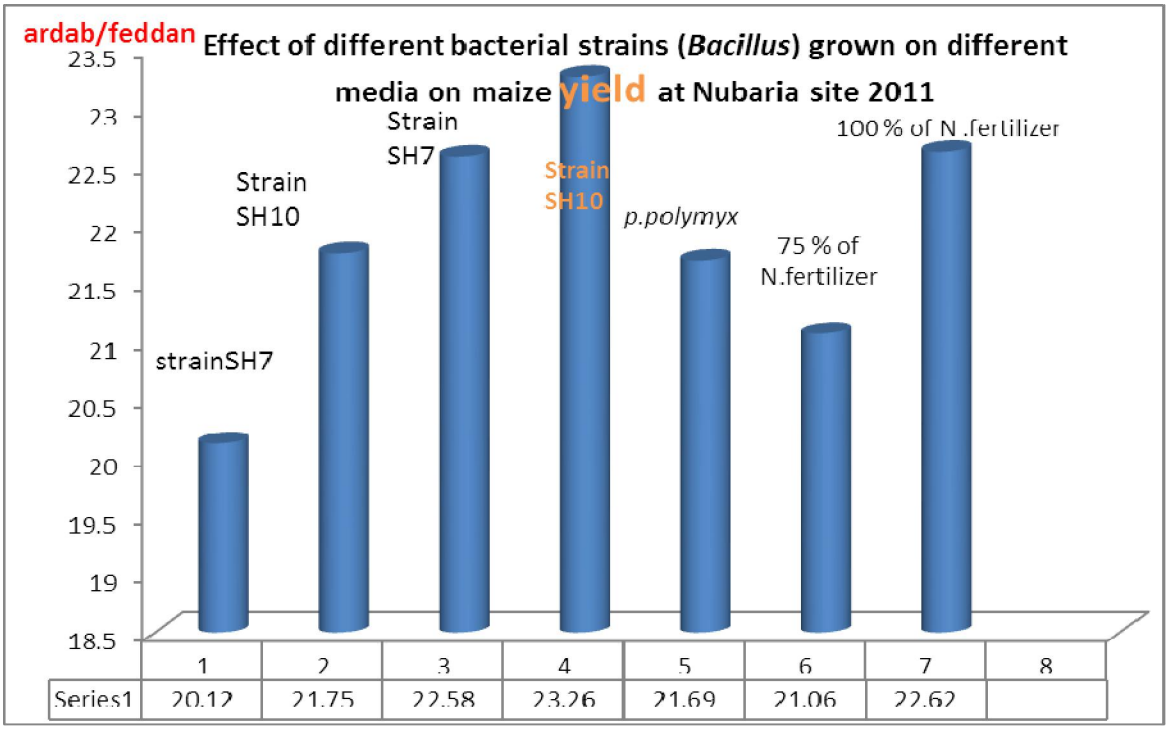

Fig 9: Effect of inoculation of two bacterial strains SH7 & SH10 and reference strain *P. polymyxa*on maize yield at El-Nubaryia site (2011)

The fig shows the effect of bacterial inoculation on the yield of maize. The data in the fig revealed that the bacterial strain SH10 that grew on LB + additives (ASP 0.4%, NaCl 0.15%, and glycerol 7 ml%) with 75 % MF gave the highest productivity.

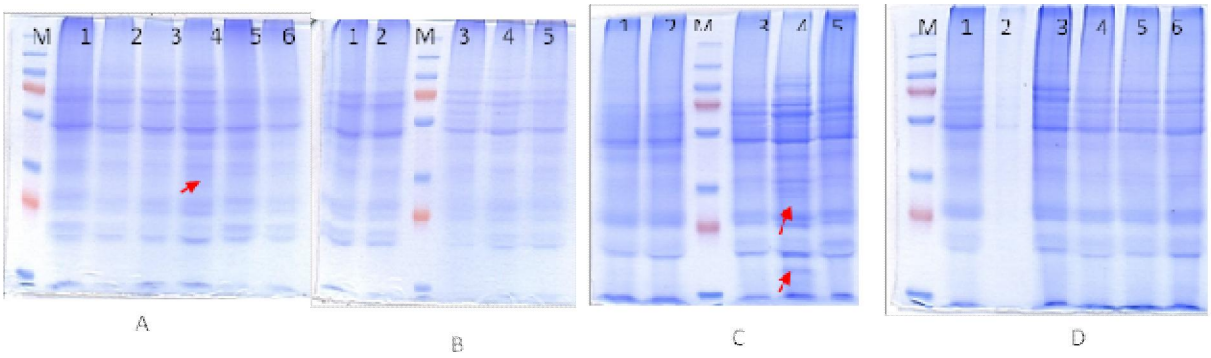

Fig 10: Total cellular protein from grind seeds of maize yield

A: lane M: protein marker, lane 1: MF 100% (R1), lane 2: SH10 (R1), lane 3: SH10 with additives (R1), lane 4: *B. polymyxa* (R1), lane 5: MF 75% (R1), lane 6: SH10 (R2). **B**: Lane 1: B. polymyxa (R2), lane 2: SH7 with additives (R1), lane M: protein marker, lane 3: SH7(R2), lane 4: SH10 with additives (R2), lane 5: MF 75% (R2).**C**: Lane 1: *SH7* (R2), lane 2: SH7 with additives (R2), lane M: protein marker, lane 3: MF 100% (R3), lane 4: SH7 (R2), lane 5: MF 75% (R3). **D**: Lane M: protein marker, lane 1: SH7 with additives (R3), lane 2: empty, lane 3: SH10(R3), lane 4: *B. polymyxa* (R3), lane 5: SH10 with additives (R3), Lane 6: SH7 with additives (R3). The protein marker that used in the 4 gels is Fermentas (Page Ruler plus prestained protein ladder) and the size of its bands from top to bottom in K Da are (250, 130, 95, 72, 55, 36, 28, 17, 11)

In Fig 10 the total protein pattern from maize seeds has been electrophoresed. These seeds coming from maize plants that inoculated with different bacterial inoculums. the strains SH7 & SH10 that

grown on LB media & that grown on LB with additives and plants inoculated with *P. polymyxa*. Samples treated with 75% and 100% MF. Three replicates (R) were used from each sample.

In (fig 10, **gel A**), one can observe the difference between lane 4 (*P. polymyxa*) and lane 1 (100% MF) & lane 5 (75% MF); lane 4 showed extra protein band slightly under the 36 KDa. In (**gel C**), it appeared the difference of protein pattern of lane 4 (SH7) from that of lane 3 (100% MF) & lane 5 (75% MF). Lan4 showed protein band in the region under the 36 KDa and that under 17 KDa (referred by red arrows).

summary: four bacterial strains were identified on molecular levels as *P. polymyxa*. GS enzyme from these strains showed high activity when the bacteria grown in LB media with additives (ASP 0.4%, NaCl 0.15%, and glycerol 7 ml%) and in presence of Mg^{++} metal ion. The four strains used in field experiments for inoculation of maize and wheat crops. The application of *P. polymyxa* bacterial strains used in this work with 75% of MF (90kg/fed.) produced an increase of: **nitrogenase activity** in soil rhizosphere of plant soil, plants inoculated with strain SH7 showed the highest nitrogenase activity (785 umol C2H4/ hr / 100g soil) that represents 94% over 100% MF. **NPK in soil and plant shoots**, plants inoculated with strain SH7 gave 84.5% N (in ppm) increase over 100% MF, plants inoculated with SH10 showed 43.5 %increase in P (in ppm) over 100% MF, plants inoculated with strain SH10 gave 60% increase in N% in plant shoots over 100% MF. **Weight of the dray shoots**, plants inoculated with strain SH7 gave increase in weight of the dray shoots of plant by 41% over that treated with 100% MF. **The plant heights**, plants inoculated with strain SH10 gave increase in plant heights by 8.6% over that treated with 100% MF. **The quality of seeds** that represented in the weight of 1000 - seed of maize, plants inoculated with strain SH10 gave 6% increase in the weight of 1000 - seed of maize more than that treated with 100% MF. **Yield**, plants inoculated with strain SH10 gave 6% increase in the yield of maize over that treated with 100% MF and 13.7% increase in the yield of wheat. **protein content** with seeds, plants inoculated with the strain SH10 showed increase in the rate of nitrogen (N) in the maize grains by 12.5% over that treated with 100% MF and consequently the protein content increased in the grains. **The protein profiling** from seeds of maize and wheat that inoculated with bacterial strains showed extra bands that not found in that treated with 100% MF. The bacterial strain SH10 showed the best results.

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